EXHIBIT K

Developmental Biology 224, 20–28 (2000)
doi:10.1006/dbio.2000.9761, available online at http://www.idealibrary.com on IDEAL®

Too Much Interference: Injection of Double-Stranded RNA Has Nonspecific Effects in the Zebrafish Embryo

Andrew C. Oates, Ashley E. E. Bruce, and Robert K. Ho[1]
Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544

We have investigated the ability of double-stranded RNA (dsRNA) to inhibit gene expression in a vertebrate, the zebrafish, *Danio rerio*. Injection of dsRNA corresponding to the T-box gene *tbx16/spadetail* (*spt*) into early wild-type embryos caused a rapid and dramatic loss of *tbx16/spt* mRNA in the blastula. mRNAs from the *papc*, *tbx6*, and *gata1* genes, which depend on *tbx16/spt* function for their expression, were reduced, apparently mimicking the *spt* mutant phenotype. However, mRNAs from a number of genes that are unaffected by the *spt* mutation, such as *β catenin*, *stat3*, and *no tail*, were also lost, indicating that the "interference" effect of *tbx16/spt* dsRNA was not restricted to the endogenous *tbx16/spt* mRNA. We compared the effects of injecting dsRNA from the zebrafish *tbx16/spadetail*, *nieuwkoid/bozozok*, and *Brachyury/no tail* genes with dsRNA from the bacterial *lacZ* gene. In each case the embryos displayed a variable syndrome of abnormalities at 12 and 24 h postfertilization. In blind studies, we could not distinguish between the effects of the various dsRNAs. Consistent with a common effect of dsRNA, regardless of sequence, injection of dsRNA from the *lacZ* gene was likewise effective in strongly reducing *tbx16/spt* and *β catenin* mRNA in the blastula. These findings indicate that, despite published reports, the current methodology of double-stranded RNA interference is not a practical technique for investigating zygotic gene function during early zebrafish development. © 2000 Academic Press

Key Words: spadetail; tbx16; no tail; nieuwkoid; dsRNA; RNAi; zebrafish; embryogenesis.

INTRODUCTION

The zebrafish has provided embryologists and developmental geneticists with an attractive system for studying the growth and organization of vertebrates, largely due to the accessibility of the embryo and the ability to isolate developmental mutations that disrupt various processes. Overexpression of mRNA encoding wild-type, activated, and dominant negative alleles has provided some information about the function of particular genes, but the construction of these variant mRNAs requires extensive knowledge of the biochemical properties of the gene product. One limitation of the zebrafish system, therefore, is the inability to disrupt the function of a gene based on sequence alone, as is possible in mice through homologous recombination in embryonic stem cells. As genomics programs in various species progress, the requirement for such technologies becomes more pressing.

[1] To whom correspondence should be addressed at the Department of Molecular Biology, Princeton University, Washington Road, Princeton, NJ 08544. Fax: (609) 258-1035. E-mail: rho@molbio.princeton.edu.

One candidate technology is the use of double-stranded RNA (dsRNA) to silence gene expression. First noted in *Caenorhabditis elegans*, this dsRNA "interference" (RNAi) relies on dsRNA homologous to a target gene as a specific means of dramatically decreasing endogenous gene expression. The biochemical mechanism of RNAi is still unclear, with recent advances including the reconstitution of interference *in vitro* (Tuschl *et al.*, 1999) and the identification of RNAi suppressor mutations in *C. elegans* and *Neurospora crassa* that likely encode RNA exonuclease and RNA-dependent RNA polymerase proteins (Cogoni and Macino, 1999; Ketting *et al.*, 1999; Smardon *et al.*, 2000). However, in practice, RNAi relies on the introduction of double-stranded RNA corresponding to a portion of a particular mRNA into the parental germ cells or the early embryo. Subsequently, the expression of the endogenous gene is perturbed; steady-state mRNA levels diminish, resulting in a concomitant decrease in the amount of encoded protein. As a result the animal expresses a complete or partial phenocopy of a null mutation of the gene in question. Introduction of sense or antisense RNA of equivalent concentration does not have this effect (but see Fire *et*

0012-1606/00 $35.00

al., 1998), whereas double-stranded intronic RNA appears to have reduced activity relative to double-stranded exonic RNA of the same length (Bosher *et al.*, 1999).

Reports of the success of RNAi in nematodes (Fire *et al.*, 1998; Guo and Kemphues, 1995; Montgomery *et al.*, 1998), fruit flies (Kennerdell and Carthew, 1998), planarians (Sanchez Alvarado and Newmark, 1999), hydra (Lohmann *et al.*, 1999), trypanosomes (Ngo *et al.*, 1998), and plants (Voinnet *et al.*, 1998; Waterhouse *et al.*, 1998) have provided the impetus to examine whether vertebrate embryos are also susceptible to these effects. Recently, claims of successful RNAi in the zebrafish (Li *et al.*, 2000; Wargelius *et al.*, 1999) and mouse (Wianny and Zernicka-Goetz, 2000) have been published.

In the present study, we sought to phenocopy the effects of the *spadetail* (*spt*) mutation of the zebrafish using dsRNA corresponding to the *spt* gene (Griffin *et al.*, 1998) first described as *tbx16* (Ruvinsky *et al.*, 1998), a member of the T-box family of transcription factors. The *spt* mutation was chosen because it acts early in development and is phenotypically and genetically well characterized (Griffin *et al.*, 1998; Ho and Kane, 1990; Kimmel *et al.*, 1989), enabling a detailed comparison with the effects of dsRNA. We show here that dsRNA injected into early zebrafish embryos causes a nonspecific depletion of several endogenous mRNAs, leading to an easily misinterpreted syndrome of developmental defects. Thus, at present, RNAi appears unsuited to application in the zebrafish embryo for the study of zygotic gene activity during development.

MATERIALS AND METHODS

Synthesis of Single-stranded (ss) and dsRNA

In all cases both ss and dsRNA for microinjection were generated from DNA templates amplified by PCR from limited regions of the cDNA of the gene in question. One or both of the primers in each primer pair contained a T7 promoter site (TAATACGACTCACTATAGGGAGG) at the 5′ end, enabling transcription directly from the PCR product, after the methods of Kennerdell and Carthew (1998). Products of the appropriate size were gel purified (GeneClean Bio101) and the Ambion mMessage mMachine and Megascript kits (Austin, TX) were used to synthesize capped and uncapped *tbx16/spt* RNA, respectively; other RNAs for injection were uncapped. Following removal of DNA template with DNase I after synthesis, RNA was purified by phenol/chloroform extraction and isopropanol precipitation, resuspended in RNase-free water, and stored at −80°C until use. Double-stranded RNA was formed either by transcribing from template with a T7 promoter at both ends or by annealing complementary ssRNA transcripts in 80 mM KCl for 2 h at 37°C after denaturation for 5 min at 70°C. The generation of ds and ss forms of RNA was confirmed by nondenaturing agarose gel electrophoresis before and after digestion with 0.5 μg/ml RNase A. RNA was diluted to the required concentration with rhodamine-conjugated dextran (Molecular Probes, Eugene, OR) in 0.2 M KCl immediately prior to injection.

To avoid nonspecific interactions with related T-box genes, a portion of the *tbx16/spt* gene which excludes the highly conserved T-box was chosen for dsRNA production. We used an 834-bp region downstream of the T-box, corresponding to nucleotides 818–1652 of the *tbx16/spt* cDNA (cDNA kindly provided by Ilya Ruvinsky, Princeton University). The *lacZ* cDNA was amplified between nucleotides 272 and 940 of the coding region to generate a 669-bp template. The *nieuwkoid/bozozok* RNA was 334 residues in length and transcribed from cDNA (kindly provided by David Koos; Princeton University) amplified between nucleotides 4 and 338 of the coding region to avoid the homeobox (Koos and Ho, 1998). *Brachyury/no tail* RNA was amplified between nucleotides 1764 and 2085 (321 bp), avoiding the T-box as described (Li *et al.*, 2000). The sequences of the primers used are *tbx16/spt* 5′ GAGATGTCCAGCCGTCATCG, *tbx16/spt* 3′ GTTAGTGCGTCCTCTCACAG; *lacZ* 5′ GGCAGATGCACGGTTACGATG, *lacZ* 3′ CCACCGCACGATAGAGATTCG; *nwk/boz* 5′ GCAACTCAAGAAGTTTTCAAA, *nwk/boz* 3′ CGCTGAGCGATTGTGTGGTA; *Bra/ntl* 5′ TTGGAACAACTTGAGGGTGA, *Bra/ntl* 3′ CGGTCACTTTTCAAAGCGTAT.

Microinjection into Zebrafish

For the majority of experiments described here, RNA was introduced into one cell of zebrafish embryos at the two-cell stage by pressure injection under an Zeiss Axioskop compound microscope (Carl Zeiss, NY); in the remainder, the RNA was injected into one cell at the one- or four-cell stages. In order to control the injection volume, rhodamine-conjugated dextran (Molecular Probes) was used to dilute the RNA preparations so that the injected bolus of approximately 0.5 nl could be visualized. dsRNA was delivered in quantities ranging from 5 fg to 100 pg per embryo. At 100 pg dsRNA per embryo, survival was compromised, whereas at 40 pg, despite developmental abnormalities, survival was normal at 24 h postfertilization (hpf). Therefore injection of 40 pg RNA was used as a standard in experiments to compare the effects of *tbx16/spt*, *nwk/boz*, *Bra/ntl*, and *lacZ* RNA. No qualitative difference in phenotype was observed between dsRNA formed by cotranscription from a single template and dsRNA formed by subsequent annealing of complementary ssRNA. To study the effects of the previously published *Bra/ntl* dsRNA (Li *et al.*, 2000) in our hands, *ntl* RNAs were injected at 5 fg (approx 10^5 molecules) and 40 pg per embryo.

Whole-Mount in Situ Hybridization

Whole-mount *in situ* hybridization was performed essentially as described (Thisse *et al.*, 1993). DNA template for a riboprobe to detect endogenous *spt* mRNA without interference from the dsRNA construct was generated from nucleotides 82–814 of the *spt* cDNA, corresponding to the T-box encoding region, with the primers *tbx16/spt* probe 5′, ATGCAGGCTATCAGAGACC, and *tbx16/spt* probe 3′, TAATACGACTCACTATAGGGAGGGGCTTCCATCTGTAGACTCT, which contains the T7 promoter sequence at the 5′ end. Probes to detect the *gata1*, *tbx6*, *papc*, *ntl*, *stat3*, and *β catenin* transcripts were synthesized as previously described (Detrich *et al.*, 1995; Hug *et al.*, 1997; Kelly *et al.*, 1995; Oates *et al.*, 1999b; Schulte-Merker *et al.*, 1994; Yamamoto *et al.*, 1998). After hybridization, embryos were mounted in glycerol and photographed on Kodak Royal Gold 100 film (Kodak, NY) using either an Olympus SZ-60 (Olympus America, NY) dissecting or a Zeiss Axioskop compound microscope. Images were scanned from print and assembled in Adobe PhotoShop.

Detection of Cell Death

Cell death was determined by uptake of acridine orange dye (Sigma A6014) into the blastoderm as described (Furutani-Seiki e

al., 1996) using the FITC filter set on a Zeiss Axioskop (Carl Zeiss) compound microscope.

RESULTS

Previous successful dsRNA experiments in *C. elegans* and *Drosophila melanogaster* utilized short stretches of exonic sequence. Likewise, we generated three RNA preparations corresponding to the coding sequence for the divergent C-terminus of the Tbx16/Spt protein: single-stranded sense and antisense RNA and an annealed dsRNA. The structure of the RNA and its relation to *tbx16/spt* mRNA are diagrammed in Fig. 1a. The presence of single- or double-stranded RNA was confirmed by RNase A digestion and gel electrophoresis (data not shown). These synthetic RNAs were introduced into one cell of the one- to four-cell blastula using standard microinjection techniques and the embryos grown for assay. We looked for the earliest effects of the injection by measuring the levels of the target *tbx16/spt* mRNA when it first appears in the embryo (Ruvinsky *et al.*, 1998). Endogenous *tbx16/spt* mRNA was absent from large sector-like areas of the blastula after midblastula transition (1000 cells) following dsRNA injection (Fig. 1b). This contrasts with widespread loss of *tbx16/spt* mRNA in *spt* mutant embryos at the same stage (Fig. 1c; Griffin *et al.*, 1998) and suggests that the diffusion of dsRNA may be constrained within the early-cleavage-stage embryo. The injection of either single-stranded *tbx16/spt* RNA preparation had no effect on endogenous *tbx16/spt* levels (Fig. 1d), and the presence of a 5′ cap analog did not alter the response of the embryo to *tbx16/spt* dsRNA or ssRNA (data not shown). The effect of *tbx16/spt* dsRNA was dose dependent, with effective depletion of *tbx16/spt* mRNA resulting from injection of as little as 0.4 pg dsRNA and the majority of injected individuals displaying an effect with 40 pg dsRNA (Fig. 1e). The cells of the blastula show no increase in cell death upon injection with *tbx16/spt* dsRNA as measured by increased uptake of acridine orange (data not shown). Since the injection of 40 pg dsRNA did not affect survival at 24 hpf, and yields a molarity within the range described by Kennerdell and Carthew (1998) as similarly effective in *Drosophila*, we chose this quantity to investigate further the effects of the treatment.

The most obvious consequence of the known mutant *spt* alleles is that cells normally fated to become paraxial mesoderm and contribute to the trunk somites fail to migrate correctly during gastrulation instead being taken up into the tailbud and incorporated in a prominent mass in the tail tip (Kimmel *et al.*, 1989). Examination of the morphology of embryos injected with *tbx16/spt* dsRNA revealed an apparent mosaic phenocopy of *spt*. At early segmentation stages, there was a unilateral loss of anterior somites in otherwise normal animals (Fig. 1f), consistent with unequal segregation of RNA between cleavage stage blastomeres. Observation of segmental boundaries in embryos at 26 hpf revealed a loss and/or reduction in anterior somite number and size as well as twisted, foreshortened tails (data not shown). We did not observe the characteristic "spadetail" of *spt* mutants in *tbx16/spt* dsRNA-injected embryos. However, we reasoned that this structure is the result of the misrouting of all trunk somites into the tail and would not be expected from the relocation of, at most, several somites as might be observed in this case. Furthermore, the *tbx16/spt* dsRNA-injected embryos were defective in the production of primitive erythrocytes, as is the *spt* mutant (Thompson *et al.*, 1998), determined by assaying for the expression of the red blood cell marker *gata1* (Fig. 1g).

In order to correlate the spatial extent of the defects caused by depletion of *tbx16/spt* message in sector-like regions of the blastoderm with the morphological defects observed after gastrulation, we compared the molecular consequences of dsRNA injection to those of the *spt* mutation around the onset of gastrulation (approximately 32,000 cells). We determined the expression of the *paraxial protocadherin* (*papc*) and *tbx6* gene in shield stage embryos after *tbx16/spt* dsRNA injection, since both these genes depend on a functional *tbx16/spt* gene for their expression (Hug *et al.*, 1997; Yamamoto *et al.*, 1998). Both *papc* and *tbx6* were depleted from arcs of the gastrula margin (Fig. 1h). These results suggest that the loss of *papc* and *tbx6* mRNAs occurs with a geometry (arcs at the margin) similar to that of the prior loss of *tbx16/spt* mRNA (sectors of the blastoderm) and are consistent with the placement of their expression downstream of the function of the Tbx16/Spt protein. Thus, multiple molecular and morphological aspects of the *spt* phenotype appear to be reproduced in a mosaic manner by introduction of *tbx16/spt* dsRNA into the embryo.

Examination of the embryos after 24 hpf, however, revealed a variable range of phenotypic consequences not seen in *spt* mutants, in addition to the effects described above. A proportion of injected embryos displayed cyclopia, with a fusion of the eyes across the anteriormost aspect of the neural tube (see Figs. 3g–3i, below). This phenotype is not seen in known *spt* alleles, despite strong expression of *tbx16/spt* mRNA in the prechordal plate (Ruvinsky *et al.*, 1998). Other novel defects include partial loss of notochord and reduced head structures (see Figs. 3j–3o, below). One explanation for these effects is that the existing *spt* mutations may be hypomorphic variants and that complete removal of *tbx16/spt* mRNA from the embryo by *tbx16/spt* dsRNA may reveal additional functions. Alternatively, *tbx16/spt* dsRNA might possess nonspecific activity with respect to endogenous mRNA.

We tested the specificity of the effects of *tbx16/spt* dsRNA by examining the expression of the T-box family member *Brachyury/no tail* (*Bra/ntl*) at early gastrula stages, since in spt mutants, *Bra/ntl* expression is unaffected in the gastrula margin (Fig. 2a). Strikingly, *Bra/ntl* mRNA was depleted from sectors of the gastrula margin by *tbx16/spt* dsRNA injection (Fig. 2b). This indicates that the *tbx16/spt* dsRNA affected another T-box family member and led us to suspect that it may affect endogenous RNAs regardless of

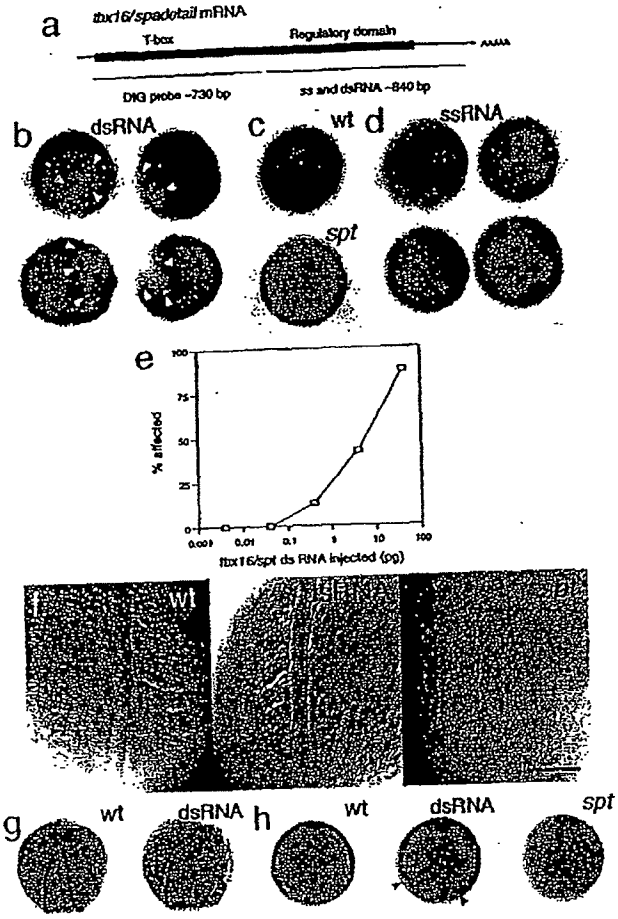

FIG. 1. *tbx16/spadetail* dsRNA injection into zebrafish embryos appears to cause a mosaic phenocopy of the *spadetail* mutation. Embryos in (b–d, h) are viewed from the animal pole, and those shown in (f, g) are viewed from the dorsal side. (a) Regions of the *spadetail* mRNA used to produce nonoverlapping antisense riboprobe and ss or dsRNA transcripts. (b–d) Expression of endogenous *spt* mRNA in response to injection of *tbx16/spt* dsRNA. Embryos are shown in animal pole view at the 40% epiboly stage after *in situ* hybridization with a riboprobe specific for the portion of the *tbx16/spt* transcript encoding the T-box. (b) Embryos injected with 40 pg *tbx16/spt* dsRNA; sectors devoid of endogenous *spt* mRNA are highlighted with arrowheads. (c) Expression of endogenous *spt* in the wild-type embryo (wt) and widespread loss in the *spt* mutant embryo (*spt*) at 40% epiboly. (d) Endogenous *tbx16/spt* expression at 40% epiboly is unaffected by injection of antisense *tbx16/spt* ssRNA corresponding to the same region of the mRNA used to generate dsRNA. (e) Dose response to *tbx16/spt* dsRNA injection measured by depletion of endogenous *tbx16/spt* mRNA at 40% epiboly. Different concentrations of RNA were injected into embryos at a constant volume. Between 20 and 56 embryos were used for each concentration point. (f) Formation of anterior trunk somites in response to tbx16/spt dsRNA. Embryos at the 3-somite stage, showing normal somite boundary formation, denoted with arrowheads in wild-type, uninjected embryos (wt), unilateral loss of anterior somites in *tbx16/spt* dsRNA-injected embryos (dsRNA), and complete lack of somites in *spt* mutant embryos (*spt*). (g) Expression of red blood cell marker *gata1* in 10-somite embryos, showing normal primitive blood in wild-type, uninjected embryos and a depletion of *gata1*-expressing cells along the lateral plate mesoderm (arrowheads) in *tbx16/spt* dsRNA-injected embryos. (h) Expression of prospective paraxial mesoderm marker *papc* at 50% epiboly with dorsal to the top, showing radial expression in the margin in wild-type, uninjected embryos, disrupted marginal expression in *tbx16/spt* dsRNA-injected embryos, and an absence of *papc* in the margin in *spt* mutant embryos.

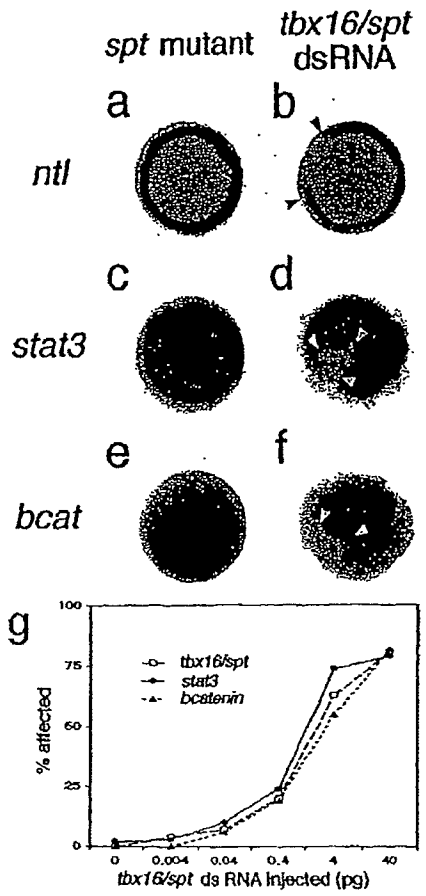

FIG. 2. The effects of *tbx16/spadetail* dsRNA injections are not restricted to those seen in the *spadetail* mutant. Embryos are viewed from the animal pole at 40% epiboly after *in situ* hybridization with *ntl* [a, b], *stat3* [c, d], or *β catenin* riboprobe [e, f]. Embryos from *spt* mutant clutches exhibit gene expression patterns identical to those of wild type [a, c, e], whereas injection with 40 pg *tbx16/spt* dsRNA causes depletion of the message from sector-like regions of the blastoderm [b, d, f]. [g] Concentration dependence of the depletion of endogenous *tbx16/spt*, *stat3*, and *β catenin* mRNA at 40% epiboly on the amount of injected *tbx16/spt* dsRNA, measured by *in situ* hybridization.

their sequence. We therefore tested mRNA levels from the *β catenin* and *stat3* genes, which are structurally unrelated to the T-box family. We observed depletion of *β catenin* and *stat3* in *tbx16/spt* dsRNA-injected embryos in sector-like regions of the blastoderm (Figs. 2d and 2f), whereas the *spt* mutation has no effect on the levels of either of these mRNAs (Figs. 2c and 2e). Thus the ability of *tbx16/spt* dsRNA to deplete mRNA from the blastula is not restricted to a single target mRNA.

To test whether there existed an amount of dsRNA at which the effects on the target *tbx16/spt* mRNA were specific, we injected *tbx16/spt* dsRNA over a wide concentration range and measured the mRNA levels at 40% epiboly of the *tbx16/spt*, *stat3*, and *β catenin* genes. The mRNAs of *stat3*, *β catenin*, and *tbx16/spt* were depleted with equal efficacy by *tbx16/spt* dsRNA across a concentration range from 0.004 to 40 pg per embryo (Fig. 3g). These results suggest that the *tbx16/spt* dsRNA preparation caused a nonspecific depletion of multiple endogenous mRNAs, instead of a specific, targeted event.

We tested the specificity of dsRNA treatments further by comparing the effects of *tbx16/spt* dsRNA injection with the injection of dsRNA corresponding to the *lacZ* gene of *Escherichia coli* for which there is no endogenous counterpart in zebrafish. The structure of the dsRNA and its relation to *lacZ* mRNA are diagrammed in Fig. 3a. We first assessed the effect of *lacZ* dsRNA species on endogenous mRNA. Expression of *tbx16/spt*, *ntl*, *stat3*, and *β catenin* mRNA after injection of *lacZ* dsRNA was depleted in a manner indistinguishable from that caused by the *tbx16/spt* dsRNA (Figs. 3b and 3c). Injection of sense or antisense versions of the *lacZ* RNA at equivalent concentrations had no effect on endogenous mRNA (Figs. 3d and 3e). This suggests that the presence of dsRNA itself causes a depletion of endogenous mRNA irrespective of the sequence of the exogenous double-stranded material.

Introduction of *lacZ* dsRNA by microinjection at the most effective dose for *spt* dsRNA (40 pg) caused a range of phenotypic consequences after 24 hpf, whereas the single-stranded sense or antisense versions of this RNA were without consequence. The phenotype of these *lacZ* dsRNA-injected embryos was highly reminiscent of that seen with *tbx16/spt* dsRNA with defects in both head and tail of the developing embryo (Fig. 3f). Indeed, in blind control experiments, we were unable to distinguish the morphological effects of *lacZ* dsRNA injection from the effects of *tbx16/spt* (Fig. 3f) or from *nieuwkoid/bozozok* dsRNA (*nwk/boz*; another early acting zebrafish gene (Koos and Ho, 1999); not shown). Defects in the head included cyclopia (Figs. 3g–3i), reduced brain structures, and marked asymmetries in the eyes (Figs. 3j–3k). Posterior structures were also affected, including a failure to form anterior trunk somites; twisted foreshortened tails, and partial loss of notochord (Figs. 3m–3o).

Finally, we tested whether a dsRNA corresponding to the *Bra/ntl* gene could produce a distinct developmental defect, as has been recently suggested (Li *et al.*, 2000; Wargelius *et al.*, 1999). We synthesized ss and dsRNA in accordance with a recently published description of potent, specific RNAi activity from the *Bra/ntl* gene (Li *et al.*, 2000). Injection of

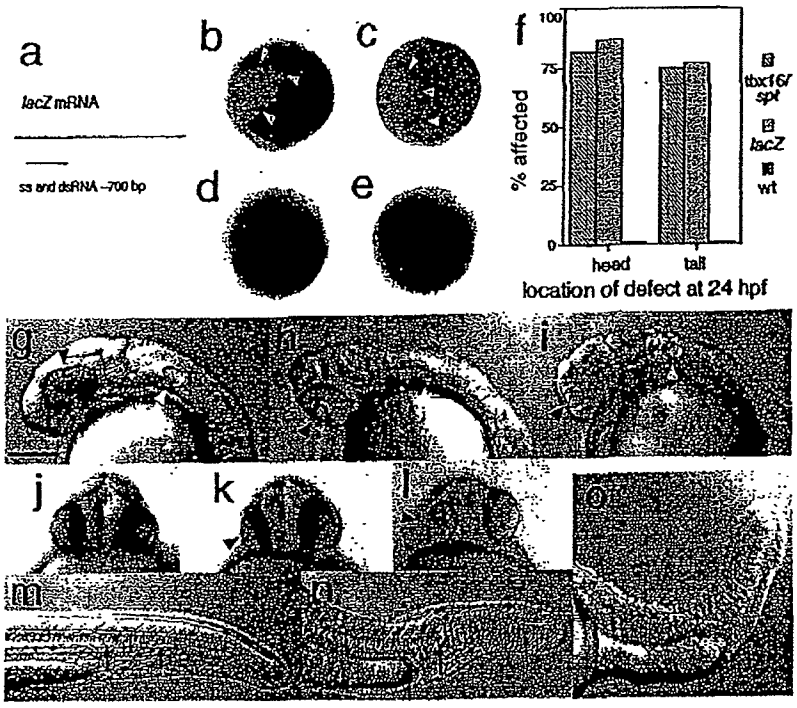

FIG. 3. dsRNA causes nonspecific depletion of mRNA. Embryos are viewed from the animal pole in (b–e), from a lateral perspective with anterior to the left in (g–i) and (m–o), and from a rostral perspective in (j–l). Scale bar for (g–o) is 250 μm. (a) Region of the *E. coli lacZ* mRNA used to produce sense, antisense, and dsRNA preparations. (b–e) Expression patterns of endogenous *tbx16/spt* and *β catenin* in embryos at 40% epiboly after injection of either ss or dsRNA from the *lacZ* gene. *spt* (b) and *β catenin* (c) expression after *lacZ* dsRNA injection was depleted from sector-like regions of the blastoderm in a manner identical to that seen after 40 pg *tbx16/spt* dsRNA injection. Injection of sense (d) or antisense (e) *lacZ* RNA did not affect the embryos. (f) Comparison of the frequency of developmental defects at 26 hpf to head or tail of the embryo after injection of *tbx16/spt* or *lacZ* dsRNA. At 26 h after dsRNA injection the embryos were scored for defects in anterior structures (head), such as cyclopia (g–i), asymmetric eyes (j–l), or small or disorganized brain, and for posterior structures (tail), such as reduced anterior somites, or twisted foreshortened tails, and partial loss of notochord (m–o). Embryos in (h, k, n) have been injected with 40 pg *lacZ* dsRNA and embryos in (i, l, o) with *tbx16/spt* dsRNA. Between 22 and 54 (mean of 32) embryos were examined for each treatment. Black arrowheads in (g–i) indicate eyes and white arrowheads the ear. In (k, l), black arrowheads indicate the smaller, abnormal eye. The loss (n) or defective structure (o) of the notochord and the resulting abnormal somite shape are indicated with arrows in (n, o) and, for comparison, the same region of an uninjected embryo in (m).

Bra/ntl dsRNA at doses of approximately 0.005 pg (5×10^5 molecules) per embryo had no phenotypic consequences, in contrast to published data. However, introduction of *Bra/ntl* dsRNA, but not ssRNA, at 40 pg per embryo yielded a syndrome of developmental defects, including the failure to form anterior trunk somites, cyclopia, foreshortened tails, and reduced head structures, which was indistinguishable from that seen with injection of *lacZ* dsRNA (Figs. 3g–3o). In summary, our results indicate that current technologies for dsRNA production and introduction into the early embryo are not effective methods to investigate zygotic gene function in the zebrafish.

DISCUSSION

We have investigated the ability of dsRNA to cause a specific phenocopy of a zygotic mutation in the zebrafish and have found no evidence of such activity. Previously, dsRNA has been widely and successfully used in inverte-

brates and plants to inhibit the expression of a number of specific genes [for recent review, see Bosher and Labouesse, 2000]. These reports raised the exciting possibility that dsRNA might be a universal mechanism for specifically perturbing gene expression. In light of our results, we stress that a technique for investigating the biological function of unknown genes must be sufficiently sensitive to distinguish between the functions of closely related homologous genes. It must also be sufficiently reliable to enable robust conclusions to be drawn; that is, it must work for most genes, if not all. Finally, its employment should be sufficiently straightforward so as to enable multiple laboratories to become proficient and thus to repeat and extend each other's results.

It is clear from our results that the morphological defects caused by the introduction of different dsRNA molecules into the zebrafish cannot be distinguished from one another. It was not possible to determine whether an embryo had been injected with dsRNA corresponding to the endogenous *tbx16/spt*, *Bra/ntl*, or *nwk/boz* gene, despite the dramatic differences in phenotype displayed by animals carrying a loss-of-function mutation in one of these genes. Furthermore, no morphological or molecular distinction was found between these treatments and the delivery of dsRNA derived from the bacterial *lacZ* gene. This overlap in effect appears due to a nonspecific depletion of multiple mRNA species in the cells of the blastula by the dsRNA. The depleted endogenous mRNA could, in principle, be either degraded or sequestered from detection by hybridization, but our experiments do not address the mechanism of this loss. We saw no depletion of endogenous mRNAs after injection of ssRNA, and although ssRNA preparations may contain dsRNA at levels that induce RNAi [Guo and Kemphues, 1995], our titration experiments argue that dsRNA was not present in our ssRNA preparations above 1 part in 1000.

It is possible that a gene-specific effect might be produced, but be masked by the nonspecific effects seen at dsRNA concentrations sufficient to perturb morphology or deplete endogenous mRNAs. Unless some way can be found to suppress the nonspecific effects of dsRNA while retaining this putative specific activity, perhaps by a modification of dsRNA structure, this effect will be of little use. Alternatively, there may be a window of dsRNA concentration in which specific effects predominate. Our results suggest that this window would be small and that the proportion of specifically affected embryos would likewise be dauntingly low. We suggest that if the result of *tbx16/spt* and *lacZ* dsRNA injections cannot be distinguished, the reliable determination of function for genes with unknown activity would pose a considerable challenge.

We note that the range of defects observed as a result of dsRNA injection overlapped with those expected from a *spt* mutant. Careful examination and controls were required to distinguish the syndrome observed from a *bona fide* mosaic phenocopy. The effect of dsRNA on endogenous mRNA was confined to a sector of the blastoderm not larger than one-quarter, contrasting with the wider distribution of single-stranded RNA across the blastoderm seen from standard microinjections. It is possible that some mechanism actively sequesters the injected dsRNA. The cells in the affected sector appeared to have lost some or all mRNA, but not to have died before the onset of gastrulation. We propose that cells in the affected sector are developmentally unresponsive for the length of time required to overcome the dsRNA perturbation; they would not be expected to divide, migrate, or differentiate with the correct timing. If a sector of these cells is created before gastrulation, the cells would be unable to participate actively in the movements of gastrulation and may subsequently be locally dispersed, leading to an embryo mosaic for the delayed cells. Importantly, the regions of the embryo that would normally have been derived from these cells will be missing or severely compromised. Thus any mutation that causes a defect during gastrulation could potentially be phenocopied, in part, by this treatment if the sector of mRNA-deprived cells were to be positioned by chance in the appropriate region of the blastula. For example, if the inert sector of cells were positioned in the prospective somite field of the late blastula, these cells would not be competent to differentiate as paraxial mesoderm and thus would not contribute to the anterior somites on one side of the embryo, apparently phenocopying the *spt* mutation. We note that the defects observed in embryos after 24 hpf illustrated in Fig. 3 resemble previously characterized mutant phenotypes. These include *cyclops* in 3g–3i [Hatta *et al.*, 1991] and *no tail* in 3m–3o [Halpern *et al.*, 1993; Li *et al.*, 2000; Wargelius *et al.*, 1999], and a reduction in eye size [3j–3l] could be attributed to interference in, for example, *pax6* function [Li *et al.*, 2000]. Simple controls involving accurate measurement of multiple endogenous mRNA species must be performed to avoid this potential confusion.

Why does this technique appear to be so successful in flies and worms and other organisms but to fail in zebrafish? One reason is that zebrafish cells may treat the dsRNA as a warning sign of viral infection. Until very recently, the primary cellular response to dsRNA was understood to be a profound physiological antiviral reaction, involving interferon-dependent and -independent pathways [for review, see Kumar and Carmichael, 1998]. In mammalian cells, the presence of cytoplasmic dsRNA triggers the activation of the 2′,5′-oligoadenylate synthetase/RNase L pathway, which can cleave both viral and cellular ssRNA, and the induction of the synthesis of interferons. It is possible that we have activated a similar mechanism in the zebrafish embryo. In support of this possibility, we note that homologs of genes involved in the regulation of RNase L are abundant in zebrafish EST databases [Accession Nos. AW510273, AW422162, *et al.*], that interferon- and dsRNA-inducible *Mx* genes have been identified in the Atlantic salmon [Robertsen *et al.*, 1997], and that the components of the interferon signal transduction pathway are present in the early zebrafish embryo [Conway *et al.*, 1997; Oates *et al.*, 1999a,b]. These studies suggest

that the early zebrafish embryo may provide a model for the study of these responses. However, if RNAi is to work generally in vertebrates, we suggest that methods to avoid this response may have to be found, perhaps by co-injecting inhibitors of the 2′,5′-oligoadenylate synthetase/RNase L pathway. Another clue to the differences in response to dsRNA between invertebrates and the zebrafish may come from recent findings that the genes involved in RNAi in the nematode are normally required for the suppression of transposon mobilization [Ketting *et al.*, 1999; Tabara *et al.*, 1999]. Although distant relatives of these genes are present in vertebrate genomes, the threat to genomic stability posed by transposon mobilization may not be as acute in vertebrates, and these genes may have evolved different functions and so possess altered biochemical activities.

In conclusion, the RNAi methods we have employed to perturb zygotic gene function in the zebrafish, despite their similarity to published protocols, have failed to produce specific effects on endogenous mRNAs or embryogenesis. We have shown that a nonspecific depletion of multiple endogenous mRNA species is caused by the introduction of dsRNA, independent of the sequence of the exogenous material, and suggest that extreme caution must be exercised when interpreting phenotypes produced by dsRNA in zebrafish.

ACKNOWLEDGMENTS

Thanks to Tracy Roskoph for fish care and to Mary Ellen Lane, Martin Lackmann, Shuo Lin, and Steve Ekker for discussions on the technique. Work in this lab was supported by a Ludwig Institute for Cancer Research Postdoctoral Fellowship to A.C.O., by NRSA 5F32GM19091-04 to A.E.E.B., and by NIH RO1 HD34499 and NSF IBN9808351 to R.K.H., who is a Rita Allen Foundation Scholar.

REFERENCES

Bosher, J. M., Dufourcq, P., Sookhareea, S., and Labouesse, M. (1999). RNA interference can target pre-mRNA: Consequences for gene expression in a Caenorhabditis elegans operon. *Genetics* 153, 1245–1256.

Bosher, J. M., and Labouesse, M. (2000). RNA interference: Genetic wand and genetic watchdog. *Nat. Cell Biol.* 2, E31–E36.

Cogoni, C., and Macino, G. (1999). Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. *Nature* 399, 166–169.

Conway, G., Margoliath, A., Wong-Madden, S., Roberts, R. J., and Gilbert, W. (1997). Jak1 kinase is required for cell migrations and anterior specification in zebrafish embryos. *Proc. Natl. Acad. Sci. USA* 94, 3082–3087.

Detrich, H. W., 3rd, Kieran, M. W., Chan, F. Y., Barone, L. M., Yee, K., Rundstadler, J. A., Pratt, S., Ransom, D., and Zon, L. I. (1995). Intraembryonic hematopoietic cell migration during vertebrate development. *Proc. Natl. Acad. Sci. USA* 92, 10713–10717.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811.

Furutani-Seiki, M., Jiang, Y. J., Brand, M., Heisenberg, C. P., Houart, C., Beuchle, D., van Eeden, F. J., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., Kelsh, R. N., Mullins, M. C., Odenthal, J., and Nusslein-Volhard, C. (1996). Neural degeneration mutants in the zebrafish, *Danio rerio*. *Development* 123, 229–239.

Griffin, K. J., Amacher, S. L., Kimmel, C. B., and Kimelman, D. (1998). Molecular identification of *spadetail*: Regulation of zebrafish trunk and tail mesoderm formation by T-box genes. *Development* 125, 3379–3388.

Guo, S., and Kemphues, K. J. (1995). *par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* 81, 611–620.

Halpern, M. E., Ho, R. K., Walker, C., and Kimmel, C. B. (1993). Induction of muscle pioneers and floor plate is distinguished by the zebrafish *no tail* mutation. *Cell* 75, 99–111.

Hatta, K., Kimmel, C. B., Ho, R. K., and Walker, C. (1991). The *cyclops* mutation blocks specification of the floor plate of the zebrafish central nervous system. *Nature* 350, 339–341.

Ho, R. K., and Kane, D. A. (1990). Cell-autonomous action of zebrafish *spt-1* mutation in specific mesodermal precursors. *Nature* 348, 728–730.

Hug, B., Walter, V., and Grunwald, D. J. (1997). *tbx6*, a Brachyury-related gene expressed by ventral mesendodermal precursors in the zebrafish embryo. *Dev. Biol.* 183, 61–73.

Kelly, G. M., Erezyilmaz, D. F., and Moon, R. T. (1995). Induction of a secondary embryonic axis in zebrafish occurs following the overexpression of *beta-catenin*. *Mech. Dev.* 53, 261–273.

Kennerdell, J. R., and Carthew, R. W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the wingless pathway. *Cell* 95, 1017–1026.

Ketting, R. F., Haverkamp, T. H., van Luenen, H. G., and Plasterk, R. H. (1999). *Mut-7* of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* 99, 133–141.

Kimmel, C. B., Kane, D. A., Walker, C., Warga, R. M., and Rothman, M. B. (1989). A mutation that changes cell movement and cell fate in the zebrafish embryo. *Nature* 337, 358–362.

Koos, D. S., and Ho, R. K. (1998). The *nieuwkoid* gene characterizes and mediates a Nieuwkoop-center-like activity in the zebrafish. *Curr. Biol.* 8, 1199–1206.

Koos, D. S., and Ho, R. K. (1999). The *nieuwkoid/dharma* homeobox gene is essential for *bmp2b* repression in the zebrafish pregastrula. *Dev. Biol.* 215, 190–207.

Kumar, M., and Carmichael, G. G. (1998). Antisense RNA: Function and fate of duplex RNA in cells of higher eukaryotes. *Microbiol. Mol. Biol. Rev.* 62, 1415–1434.

Li, Y. X., Farrell, M. J., Liu, R., Mohanty, N., and Kirby, M. L. (2000). Double-stranded RNA injection produces null phenotypes in zebrafish. *Dev. Biol.* 217, 394–405.

Lohmann, J. U., Endl, I., and Bosch, T. C. (1999). Silencing of developmental genes in Hydra. *Dev. Biol.* 214, 211–214.

Montgomery, M. K., Xu, S., and Fire, A. (1998). RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* 95, 15502–15507.

Ngo, H., Tschudi, C., Gull, K., and Ullu, E. (1998). Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. USA* 95, 14687–14692.

Oates, A. C., Brownlie, A., Pratt, S. J., Irvine, D. V., Liao, E. C., Paw, B. H., Dorian, K. J., Johnson, S. L., Postlethwait, J. H., Zon, L. I., and Wilks, A. F. (1999a). Gene duplication of zebrafish *JAK2* homologs is accompanied by divergent embryonic expression

patterns: Only *jak2a* is expressed during erythropoiesis. *Blood* 94, 2622–2636.

Oates, A. C., Wollberg, P., Pratt, S. J., Paw, B. H., Johnson, S. L., Ho, R. K., Postlethwait, J. H., Zon, L. I., and Wilks, A. F. (1999b). Zebrafish *stat3* is expressed in restricted tissues during embryogenesis and *stat1* rescues cytokine signaling in a STAT1-deficient human cell line. *Dev. Dyn.* 215, 352–370.

Robertsen, B., Trobridge, G., and Leong, J. A. (1997). Molecular cloning of double-stranded RNA inducible Mx genes from Atlantic salmon (*Salmo salar L.*). *Dev. Comp. Immunol.* 21, 397–412.

Ruvinsky, I., Silver, L. M., and Ho, R. K. (1998). Characterization of the zebrafish *tbx16* gene and evolution of the vertebrate T-box family. *Dev. Genes Evol.* 208, 94–99.

Sanchez Alvarado, A., and Newmark, P. A. (1999). Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl. Acad. Sci. USA* 96, 5049–5054.

Schulte-Merker, S., van Eeden, F. J., Halpern, M. E., Kimmel, C. B., and Nusslein-Volhard, C. (1994). *no tail* (*ntl*) is the zebrafish homologue of the mouse T (Brachyury) gene. *Development* 120, 1009–1015.

Smardon, A., Spoerke, J. M., Stacey, S. C., Klein, M. E., Mackin, N., and Maine, E. M. (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. *Curr. Biol.* 10, 169–178.

Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C. C. (1999). The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* 99, 123–132.

Thisse, C., Thisse, B., Schilling, T. F., and Postlethwait, J. H. (1993). Structure of the zebrafish *snail1* gene and its expression in wild-type, *spadetail* and *no tail* mutant embryos. *Development* 119, 1203–1215.

Thompson, M. A., Ransom, D. G., Pratt, S. J., MacLennan, H., Kieran, M. W., Detrich, H. W., 3rd, Vail, B., Huber, T. L., Paw, B., Brownlie, A. J., Oates, A. C., Fritz, A., Gates, M. A., Amores, A., Bahary, N., Talbot, W. S., Her, H., Beier, D. R., Postlethwait, J. H., and Zon, L. I. (1998). The *cloche* and *spadetail* genes differentially affect hematopoiesis and vasculogenesis. *Dev. Biol.* 197, 248–269.

Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P., and Sharp, P. A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. *Genes Dev.* 13, 3191–3197.

Voinnet, O., Vain, P., Angell, S., and Baulcombe, D. C. (1998). Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* 95, 177–187.

Wargelius, A., Ellingsen, S., and Fjose, A. (1999). Double-stranded RNA induces specific developmental defects in zebrafish embryos. *Biochem. Biophys. Res. Commun.* 263, 156–161.

Waterhouse, P. M., Graham, M. W., and Wang, M. B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl. Acad. Sci. USA* 95, 13959–13964.

Wianny, F., and Zernicka-Goetz, M. (2000). Specific interference with gene function by double-stranded RNA in early mouse development. *Nat. Cell Biol.* 2, 70–75.

Yamamoto, A., Amacher, S. L., Kim, S. H., Geissert, D., Kimmel, C. B., and De Robertis, E. M. (1998). Zebrafish *paraxial protocadherin* is a downstream target of *spadetail* involved in morphogenesis of gastrula mesoderm. *Development* 125, 3389–3397.

Received for publication March 17, 2000
Revised April 19, 2000
Accepted April 20, 2000